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Differential Requirements for Runx Proteins in *CD4* Repression and Epigenetic Silencing during T Lymphocyte Development

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Summary

T lymphocytes differentiate in discrete stages within the thymus. Immature thymocytes lacking CD4 and CD8 coreceptors differentiate into double-positive cells ($CD4^+CD8^+$), which are selected to become either $CD4^+CD8^-$ helper cells or $CD4^-CD8^+$ cytotoxic cells. A stage-specific transcriptional silencer regulates expression of CD4 in both immature and $CD4^-CD8^+$ thymocytes. We show here that binding sites for Runt domain transcription factors are essential for *CD4* silencer function at both stages, and that different Runx family members are required to fulfill unique functions at each stage. Runx1 is required for active repression in $CD4^-CD8^-$ thymocytes whereas Runx3 is required for establishing epigenetic silencing in cytotoxic lineage thymocytes. Runx3-deficient cytotoxic T cells, but not helper cells, have defective responses to antigen, suggesting that Runx proteins have critical functions in lineage specification and homeostasis of CD8-lineage T lymphocytes.

Introduction

Determination of specific cell fates during development of multicellular organisms is dependent on the activation as well as the heritable repression of genes. Stable silencing of specific genes is thought to be mediated through establishment of heterochromatin, which then allows maintenance of the repressed state through epigenetic mechanisms (Fisher and Merkenschlager, 2002; Lewin, 1998; Wolffe and Matzke, 1999). Much of our insight into epigenetic regulation of gene expression in vertebrates comes from studies of the mechanisms of X chromosome inactivation and *H19* genomic imprinting, both of which are initiated in early embryogenesis (Csankovszki et al., 1999; Srivastava et al., 2000). Much less is known as to how epigenetic gene silencing is involved in regulation of lineage choice and maintenance of terminally differentiated properties of cells at later stages of organismal development. Genes of the Polycomb group are known to have important roles in maintenance of silencing at loci such as the *Hox* genes (Akasaka et al., 2001), but how establishment of silencing of individual genes is linked to the maintenance mechanism remains poorly understood. It remains unclear, for example, whether classical transcription factors that bind to specific DNA sequences play a role in epigenetic gene silencing in vertebrates.

The *CD4* locus is, to our knowledge, the only vertebrate gene in which *cis*-acting sequences required for in vivo developmental stage and lineage-specific epigenetic silencing have been characterized (Ellmeier et al., 1999; Sawada et al., 1994; Siu et al., 1994; Taniuchi et al., 2002; Zou et al., 2001). This provides a unique opportunity to characterize the factors involved in establishment of epigenetic silencing and elucidate their mechanism of action. The *CD4* gene encodes a coreceptor molecule that plays a pivotal role during thymocyte differentiation by interacting with MHC class II molecules together with the $\alpha\beta$ T cell antigen receptors (TCRs) (Killeen and Littman, 1996). Early T lymphocyte progenitors do not express either CD4 or the CD8 coreceptor and are termed double-negative (DN) thymocytes. DN cells that undergo in-frame rearrangements of their TCRβ genes express a functional pre-TCR complex, which permits selection (termed β selection) to the next developmental stage, during which both CD4 and CD8 are expressed and the *TCR*α loci undergo rearrangement (Groettrup and von Boehmer, 1993). The $CD4^+CD8^+$ (double-positive or DP) thymocytes then undergo positive selection if their TCR$\alpha\beta$ molecules interact productively with MHC class I or class II molecules complexed to self-peptides, and they differentiate into two functionally different lineages of T lymphocytes, the $CD4^+CD8^-$ helper cells and the $CD4^-CD8^+$ cytotoxic T cells (von Boehmer et al., 1989). CD4-lineage T cells generally interact with MHC class II, while CD8-lineage cells interact with MHC class I. The mechanism by which the specificity of TCR for MHC is translated into the correct lineage choice for DP thymocytes remains a matter of considerable controversy. An understanding of the regulation of coreceptor transcription, which is tightly coupled to the functional specification of T cells, is therefore likely to provide insight into the signals involved in lineage bifurcation (Ellmeier et al., 1999).

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The *CD8α* and *CD8β* genes are regulated by multiple dispersed enhancer elements that function in concert to provide stage-specific expression (Ellmeier et al., 1999; Hedrick, 2002). In contrast, *CD4* is regulated by a combination of an enhancer, that is active at all stages of T cell development, and a silencer, that represses expression in immature DN and in mature $CD8^+$ cytotoxic lineage T cells (Ellmeier et al., 1999; Sawada et al., 1994; Siu et al., 1994). Targeted mutagenesis of the *CD4* silencer in mice has shown that it is required for repression of CD4 expression in both DN and mature CD8-lineage thymocytes (Leung et al., 2001; Zou et al., 2001). Silencing of the *CD4* locus was unaffected in mature $CD8^+$ T lymphocytes if the silencer was deleted after completion of intrathymic differentiation, indicating that silencing is epigenetically maintained in these cells (Zou et al., 2001). Several motifs required for silencer function in both DN and CD8-lineage thymocytes have been identified by targeted mutagenesis in mice (Taniuchi et al., 2002). Individual mutations resulted in variegated derepression of CD4 in mature $CD8^+$ T cells. This resembled position effect variegation (PEV) of transgene expression, a well-characterized inhibitory effect of heterochromatin (Wakimoto, 1998). The overexpression of HP-1, an evolutionarily conserved molecule that enhances the silencing effect of heterochromatin, also enhanced *CD4* silencing in mutant mice with variegated CD4 derepression (Taniuchi et al., 2002). These results reinforce the conclusion that the *CD4* locus is silenced in cytotoxic-lineage T cells through an epigenetic maintenance mechanism. In immature DN thymocytes, mutations resulted in uniform partial derepression of *CD4*, which suggested that there could be different mechanisms of silencing operating at the two developmental stages at which *CD4* is repressed (Taniuchi et al., 2002).

On the basis of studies in transgenic mice and transfected cell lines, several other putative functional sites within the *CD4* silencer have been reported, along with potential binding factors, including HES-1, SAF (silencer associated factor), and c-Myb (Allen et al., 2001; Donda et al., 1996; Kim and Siu, 1998, 1999). Roles for these sites and the proposed factors have not been confirmed in vivo, and no physiologically relevant *trans*-acting factor required for CD4 silencing has previously been reported.

The Runt domain transcription factors, Runx, comprise a family of transcriptional regulators that contain a conserved 128 amino acid Runt domain responsible for sequence-specific DNA binding. Runx proteins make heterodimeric complexes with a partner protein, PEBP2β/CBFβ in mammals and Brother and Big-brother in *Drosophila*, and bind to the DNA sequence, 5′-PuACCPuCA-3′ (Bae and Ito, 1999; Wheeler et al., 2000). Three mammalian Runt domain transcription factors, Runx1/PEBP2αB/AML1, Runx2/PEBP2αA /CBFA1, and Runx3/PEBP2αC, have been identified. Runx proteins play pivotal roles in regulating transcription in developmental pathways ranging from sex determination, eye development, hematopoiesis, and segmentation in *Drosophila* to hematopoiesis, bone development, and neurogenesis in mice (Canon and Banerjee, 2000; Ducy et al., 1997; Kania et al., 1990; Komori et al., 1997; Lebestky et al., 2000; Levanon et al., 2002; Okuda et al., 1996; Otto et al., 1997; Wang et al., 1996; Inoue et al., 2002). Mutations of *RUNX1/AML1* have been identified in acute myeloid leukemia patients (Miyoshi et al., 1991; Osato et al., 1999), and mutations of *RUNX2/CBFA1* have been demonstrated in cleidocranial dysplasia (CCD) patients (Mundlos et al., 1997). Recently, mutations of *RUNX3* have been linked with gastric cancer (Li et al., 2002). Thus, abnormalities in *RUNX* genes are potentially involved in human diseases. Genetic approaches in *Drosophila* have revealed that Runx functions both to activate and to repress transcription of different target genes in a context-dependent manner (Canon and Banerjee, 2000). However, little is known about the molecular mechanisms through which Runx proteins regulate, and in particular repress, gene expression.

In this report, we show essential roles of the Runt domain binding sites within the *CD4* silencer and of Runx transcription factors in *CD4* silencing. By using mutational analyses in mice, distinct requirements for Runx1 and Runx3 were demonstrated at the two stages of development at which the *CD4* silencer is active. Runx 1 was shown to be required to repress *CD4* expression in DN thymocytes, while Runx3 was shown to be required both for establishment of epigenetic silencing and for specification of functional cytotoxic T cells. The results provide a key link between sequence-specific transcription factors and the function of a specific developmentally regulated silencer, and identify an important in vivo target for members of the Runx family.

Results

Runx Binding Sites Are Required for *CD4* Silencing In Vivo

In earlier studies combining analyses in transfected cells and in gene-targeted mice, we identified several functionally important sites within the 434 bp CD4 silencer. We focused, in particular, on a core sequence (131–265) that conferred strong inhibitory activity in transient transfection assays in the mouse $CD4^-CD8^+$ thymoma 1200M (Taniuchi et al., 2002). The core sequence or fragments derived from it were used to generate yeast reporter strains for screening a library of mouse thymus cDNAs fused to a sequence encoding the GAL4 activation domain. This screen resulted in the cloning of a cDNA encoding the Runt domain (^{1}Met-^{252}Pro) of the transcription factor Runx1. Two 5′-PuACCPuCA-3′ consensus binding motifs for Runt domain factors were present in opposite orientation within the core sequence (Figure 1A). However, mutagenesis analyses showed distinct requirements of these two motifs both for silencer activity and for binding of *trans*-acting factors. One of these motifs (sequence ^{232}GACCACA^{238}), which we have designated as site 2, is completely required for silencer activity in both the transient transfection assay (Figure 1B) and in a transgenic reporter system (Sawada et al., 1994) (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/111/5/621/DC1), while another motif (sequence ^{248}TGTGGTG^{253}) is partially required in both assays. Further fine mutagenesis analysis indicated that the minimum sequence required for site 2 function is identical to the Runx consensus motif (Figure 1C), whereas the other site required sequences outside of the consensus binding motif for its function (Figure

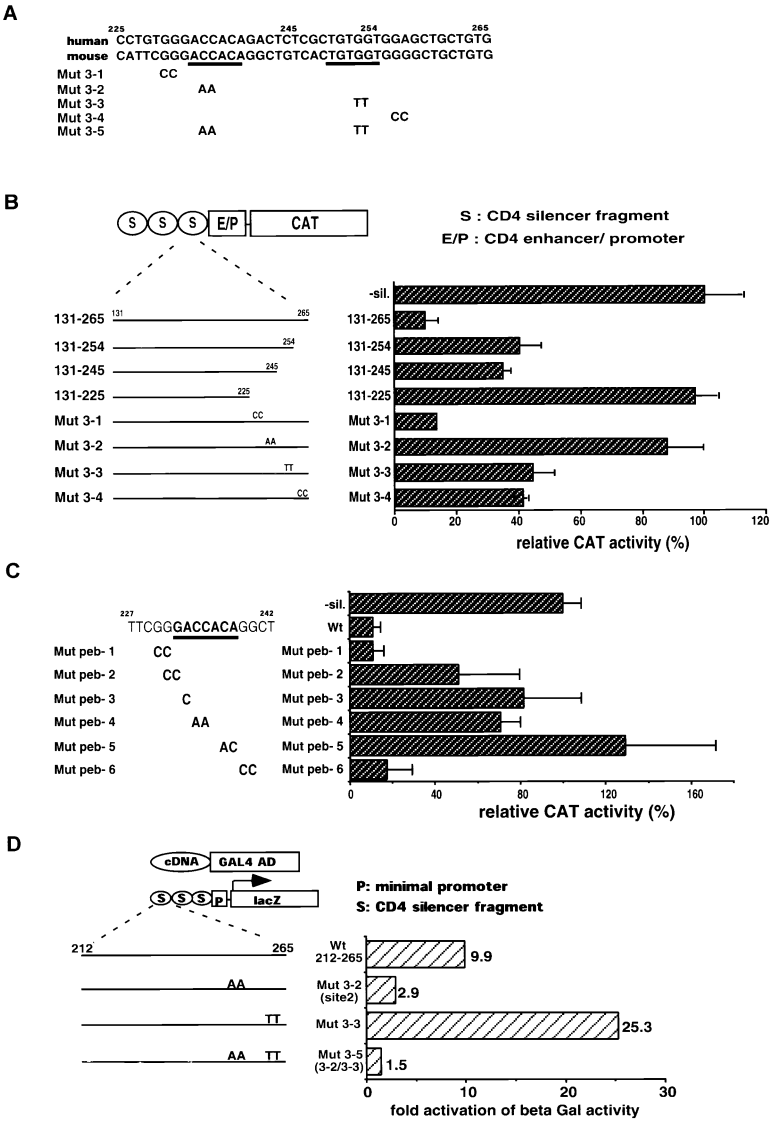

Figure 1. Requirement of a Runx Site for *CD4* Silencer-Mediated Transcriptional Repression

(A) Alignment of nucleotide sequences from the mouse and human *CD4* silencers and mutations used for reporter constructs are shown. Underlined sequences represent consensus Runx binding motifs.

(B) Effect of deletions and mutations in the putative Runx sites within the core *CD4* silencer on transcriptional repression in transiently transfected 1200M cells. Three copies of *CD4* silencer fragments (S) were inserted upstream of the *CD4* enhancer (E)/*CD4* promoter (P) in a CAT reporter construct. Results are the average from three separate experiments.

(C). Fine nucleotide sequence mapping of site 2. Transfections were as in (B). Results from three experiments were averaged with standard deviations. The underlined sequence, ^{232}GACCACA^{238}, is essential for site 2 function.

(D) Specific binding of a Runt domain to site 2 in yeast. Three copies of each *CD4* silencer fragment were inserted upstream of the minimal promoter (P) in the pLacZi vector. The fold activation of the reporter gene by the T-1 plasmid encoding a Runx1 Runt domain relative to the control plasmid (GAL4-AD only) was monitored by β-galactosidase activity in each yeast reporter strain.

1B). Consistent with this finding, the Runt domain interacted preferentially with site 2 in the yeast one-hybrid analysis (Figure 1D).

To confirm the physiological importance of the Runx binding site within the core silencer sequence in *CD4* gene regulation, we introduced mutations into the *CD4* silencer by homologous recombination in ES cells (Taniuchi et al., 2002) (Figures 2A and 2B). The ES cells were used to generate chimeric mice, and peripheral T cells expressing the ES-derived isotype marker Ly9.1 were analyzed for CD4 and CD8 expression by flow cytometry (Figure 2C). There was no derepression of CD4 in CD8$^+$ T lymphocytes from chimeric mice generated with the wild-type construct (Figure 2C). We previously showed that all mature CD8$^+$ T lymphocytes derepressed CD4 upon deletion of the entire silencer (sequences 1–429) or the core sequence (167–257) (Taniuchi et al., 2002). Mutation of site 2, the Runx binding site, resulted in variegated derepression of CD4 in 18%–30% of mature CD8$^+$ T lymphocytes (Taniuchi et al., 2002). Because we had previously observed variegated derepression of CD4 in CD8-lineage T cells following deletion of sequences 1–95 and 1–130 (Taniuchi et al., 2002), we scanned this region for additional Runx binding motifs and identified the consensus sequence, ^{81}AACCACA^{87}. Mutation of this site resulted in derepression of CD4 in about 6% of mature CD8$^+$ T lymphocytes. However, when this site was mutated in combination with site 2 in the core silencer sequence, there was full derepression of CD4 (Figure 2C). We have designated the sequence between nucleotides 81–87 as site 2′. These results indicate that sites 2 and 2′ have overlapping function and act synergistically to confer full silencing activity. They also suggest that a transcription factor that can bind to either sequence is required for *CD4* silencing.

We have previously shown that deletions or point mutations in the *CD4* silencer resulted in various degrees of CD4 derepression in immature DN thymocytes (Taniuchi et al., 2002; Zou et al., 2001). We therefore analyzed

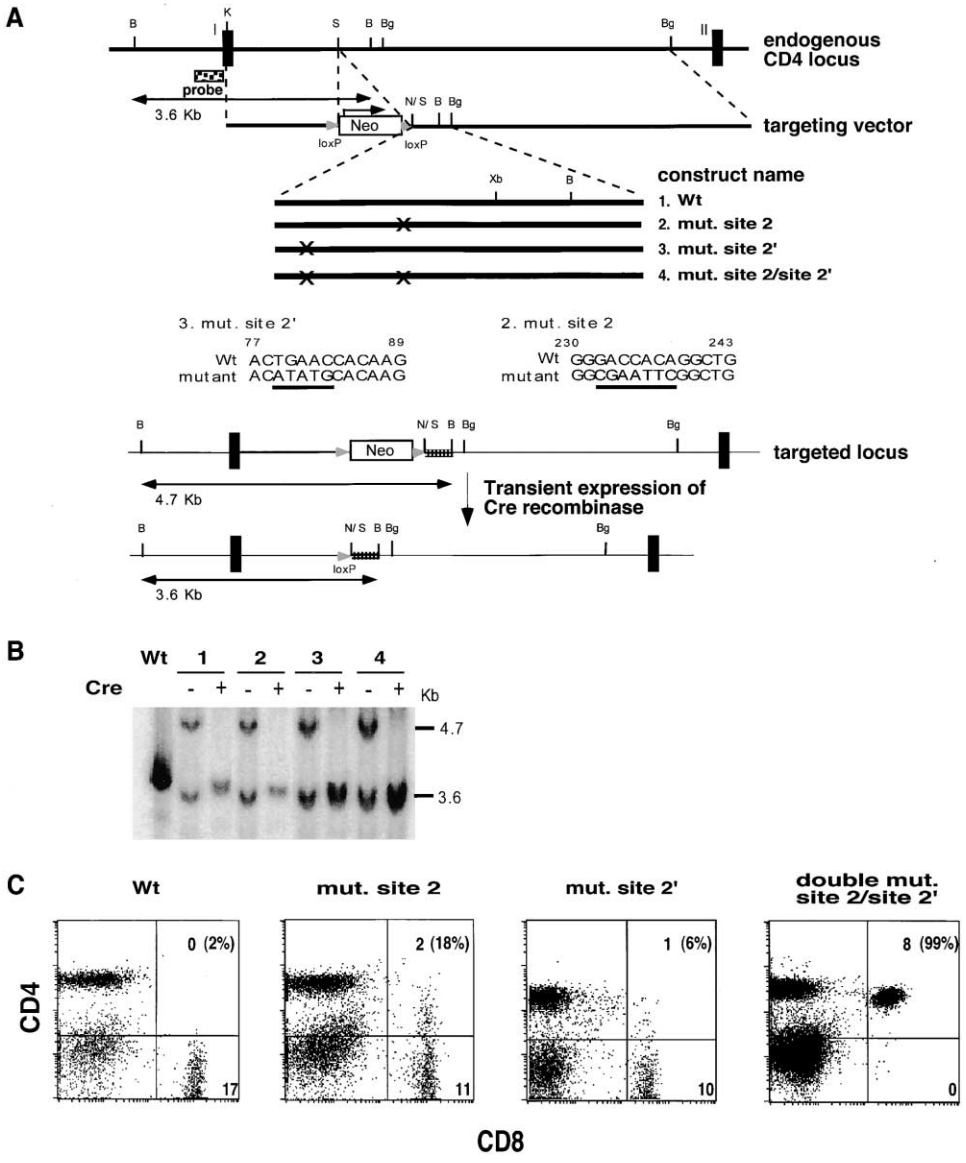

Figure 2. Runx Sites Are Required In Vivo for *CD4* Silencing in Mature CD8$^+$ T Cells

(A) Strategy for introducing mutations into the Runx sites within the *CD4* silencer at the *CD4* locus. The targeting vectors were constructed with 1.8 kb of 5′-homology region, a neomycin resistance gene (*Neo*) flanked by *loxP* sites (arrow head), a segment containing wild-type or mutant silencer, and 5 kb of 3′-homology region. Mutant sequences at site 2 and site 2' in constructs 2, 3, and 4 are underlined. The restriction sites shown are BamHI (B), KpnI (K), NotI (N), SacI (S), and XbaI (Xb).

(B) Southern blot analyses of BamHI-digested ES cell DNA hybridized with the 0.5 kb probe (shown as hatched box).

(C) Flow cytometry analyses of CD4 and CD8 expression on ES cell-derived lymphocytes from chimeric mice. Peripheral lymphocytes derived from ES cells were identified by gating for surface expression of Ly9.1. The percentage of cells that have derepressed CD4 in the CD8$^+$ population is shown in the right top corner.

the effect of the combined site 2 and 2′ mutations on CD4 expression in a population of Thy1$^+$CD8$^-$ CD3$^-$ TCR$\gamma\delta^-$ thymocytes, which correspond to DN cells. CD4 was also derepressed in these cells, in a uniform, non-variegated pattern, but its expression level was lower than observed in mice with the nucleotide 1–429 *CD4* silencer deletion (see Figure 5A, upper image). Thus, loss of the two Runx sites in the *CD4* silencer resulted in partial CD4 derepression in DN thymocytes and in full CD4 derepression in mature CD8$^+$ T lymphocytes.

Runx Proteins Actively Repress Transcription in the Context of the *CD4* Silencer

We used real-time RT-PCR to assess the level of mRNA expression for each member of the *Runx* family and for *Cbf*β at different stages of T cell development (Figure 3A). Purity of each T cell subset was monitored by *CD4* mRNA levels. Transcripts for all three *Runx* genes and for *Cbf*β were detected in each thymocyte subset and in mature T cells. However, there were marked differences in levels of expression at each stage, with Runx1

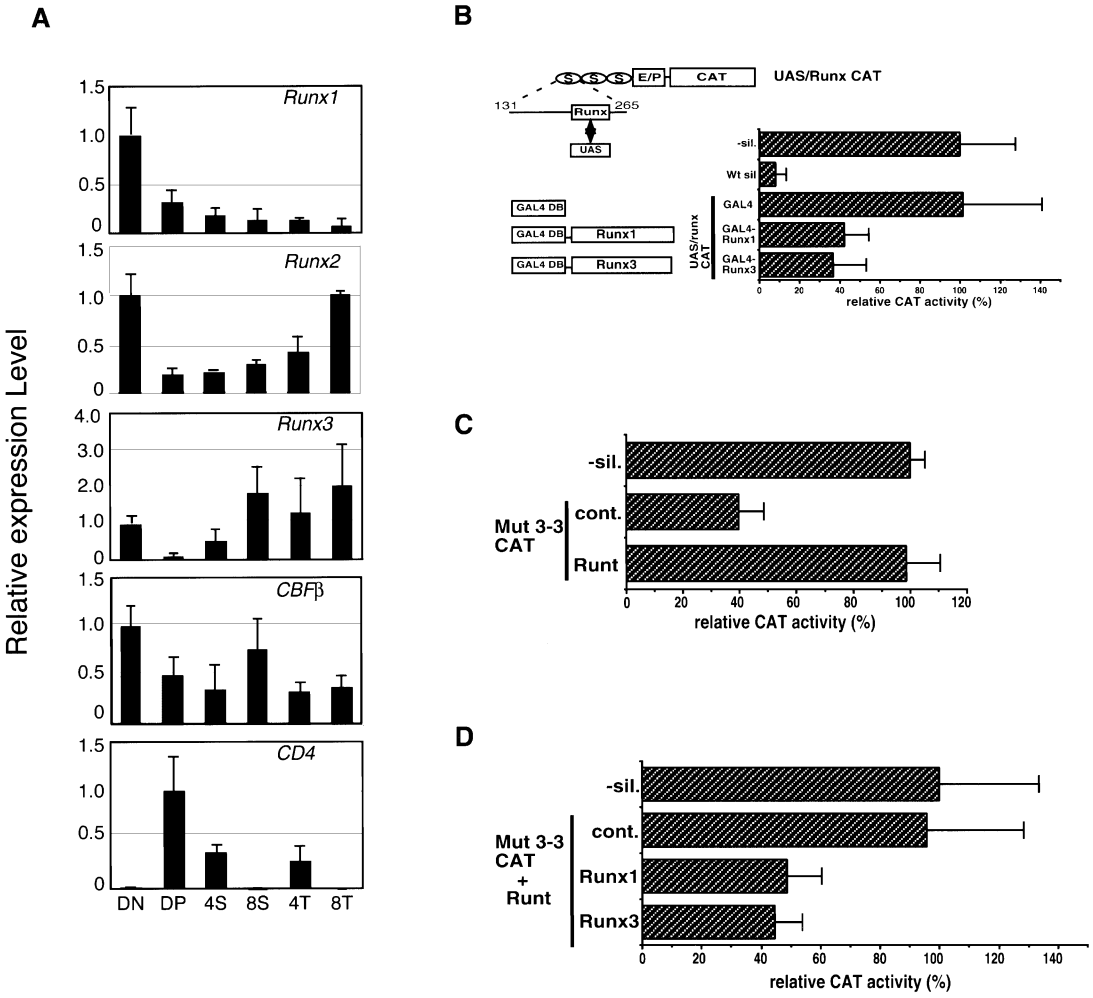

Figure 3. Expression of *Runx* Genes during T Cell Development and In Vitro Runx Repressive Activity on the *CD4* Silencer

(A) Quantitative real-time PCR of reverse-transcribed RNA from purified thymocyte and T cell subsets. The amount of transcript of the gene of interest was normalized to the amount of actin RNA in each subpopulation and is shown relative to the amount in immature $CD4^-CD8^-$ thymocytes. DN: $CD4^-CD8^-$ thymocytes; DP: $CD4^+CD8^+$ thymocytes; 4S: $CD4^+CD8^-$ thymocytes; 8S: $CD4^-CD8^+$ thymocytes; 4T: $CD4^+8^-$ splenocytes; and 8T: $CD4^-CD8^+$ splenocytes. Results from six to eight PCR reactions with two different sources of RNA were averaged with standard deviations.

(B) Repression of transcription by Runx fusion proteins in the context of the *CD4* silencer. Schematic of the UAS/Runx CAT reporter construct and GAL4-Runx1 and GAL4-Runx3 fusion proteins. The Runx site (site 2) within the 131–265 core *CD4* silencer in the CAT reporter plasmid (Figure 1B) was replaced with a GAL4 binding site (UAS). Expression vectors encoding the control GAL4 DNA binding domain (GAL4), GAL4-Runx1 or GAL4-Runx3 were transfected with the UAS/Runx CAT construct into 1200M cells. Results from three transfections were averaged with standard deviations.

(C) Inhibition of *CD4* silencer-mediated transcriptional repression by overexpression of a Runt domain. Expression vectors encoding the Runt domain (^{1}Met-^{186}Gln) of Runx1 (Runt) or control plasmid (cont.) were transfected with Mut 3-3 CAT (Figure 1B). Results from three transfections were averaged with standard deviations.

(D) Runx1 and Runx3 restore *CD4* silencer-mediated transcriptional repression impaired by Runt domain overexpression. The Mut 3-3 CAT reporter construct and the expression vector encoding only the Runt domain were cotransfected with expression plasmid encoding wild-type Runx1 (Runx1), wild-type Runx3 (Runx3), or control empty vector, pcDNA3 (cont.). Results from three transfections were averaged with standard deviations.

and Runx3 mRNAs most abundant in DN thymocytes and mature thymocytes, respectively. The level of each transcription factor did not differ significantly between thymocytes and T cells of the helper and cytotoxic lineages.

To determine whether Runx proteins act as transcriptional activators or repressors in the context of the *CD4* silencer, we examined their function in the 1200M transient transfection assay system. Proteins consisting of the GAL4 DNA binding domain (GAL4-DB) fused with Runx1 or Runx3 were both shown to repress transcription when the GAL4 binding site (UAS) was used to replace the Runx binding site within the core (131–265) *CD4* silencer fragment (Figure 3B). In addition, overexpression of the Runt domain (^{1}Met-^{186}Gln) from Runx1 resulted in the loss of residual transcriptional repression mediated by the partial loss of function mutant construct Mut 3-3 in 1200M cells (Figure 3C). Importantly, expres-

sion of full-length Runx1 or Runx3 overcame the effect of the Runt domain and restored repression (Figure 3D). Together, these results indicate that the Runx proteins can function as transcriptional repressors in the context of the *CD4* silencer.

Runx3 Is Required for Establishment of *CD4* Silencing in Mature CD8$^+$ T Cells

To verify the physiological function of the Runx proteins in *CD4* gene regulation, we analyzed strains of mice with mutations in each of the three *Runx* genes. Because null mutations of *Runx2* and *Runx3* result in neonatal lethality (Komori et al., 1997; Li et al., 2002), we analyzed T cells in *Rag2* mutant host mice, deficient for mature T lymphocytes, that had been reconstituted with fetal liver cells from *Runx* mutant embryos.

Examination of *Rag2*$^{-/-}$ mice reconstituted with fetal liver cells from *Runx2*$^{-/-}$ mice revealed normal CD4 and CD8 expression on peripheral T cells and normal T cell numbers (Figure 4A). In contrast, all host mice reconstituted with *Runx3*$^{-/-}$ progenitors had CD4$^+$CD8$^+$ peripheral T lymphocytes (Figure 4A). In most mice, CD4 was derepressed in a variegated manner in the absence of Runx3, but the degree of variegation varied in each animal. In some cases (10%), almost all peripheral CD8$^+$ T lymphocytes had derepressed CD4 (Figure 4A, right image). Since a higher percentage of peripheral CD8$^+$ T cells derepressed CD4 in host mice reconstituted with *Runx3*$^{-/-}$ progenitor cells than in mice containing single mutations of either site 2 or 2′ within the *CD4* silencer (Figure 2C), this result is consistent with binding of Runx proteins to both of these sites.

The CD4$^+$CD8$^+$ peripheral T lymphocytes from *Runx3*$^{-/-}$ reconstituted mice were mature T lymphocytes rather than immature DP thymocytes that had been released from the thymus. They expressed CD3 or TCRβ (data not shown) at the same level as CD4$^-$CD8$^+$ T cells from wild-type animals and at a significantly higher level than that on DP thymocytes (Figure 4B). They also had low expression of the differentiation marker HSA, consistent with their maturity (data not shown). Variegated derepression of CD4 was also observed in the thymus, on mature CD8$^+$ thymocytes (TCRβ^{high}/HSA^{low}) from mice reconstituted with *Runx3*$^{-/-}$ progenitors (Figure 4C).

The percentage of T lymphocytes expressing CD8, including those that had derepressed CD4, was reduced in the periphery in the absence of Runx3, resulting in an increase in the CD4:CD8 ratio to 5.8 from the normal of 2.3 (Figure 4D). In the thymus, however, the CD4:CD8 ratio among mature thymocytes (TCRβ^{high}/HSA^{low}) was not affected (Figure 4D). This discrepancy between thymus and periphery suggests that Runx3 also has a role in migration of CD8 SP thymocytes to the periphery or in homeostasis of these cells following their export.

Null mutation in *Runx1* results in loss of definitive hematopoiesis due to the absence of hematopoietic stem cells (Okuda et al., 1996). We therefore generated mice bearing *loxP*-flanked *Runx1* alleles (*Runx1*^{F}), which were bred to an *Lck-cre* transgenic strain to inactivate *Runx1* in early T cell progenitors (Supplemental Figure 2 available at http://www.cell.com/cgi/content/full/111/5/621/DC1). In contrast to the germline *Runx1*$^{-/-}$ mice, which display early embryonic lethality, *Runx1*$^{F/F}$ mice developed normally, indicating that the *Runx1*^{F} allele behaves like the wild-type gene. In thymocytes from *Runx1*$^{F/F}$*/Lck-cre* mice, more than 95% of the targeted *Runx1* alleles were deleted, allowing for analysis of the role of this gene in T cell development (Figure 4E).

In *Runx1*$^{F/F}$*/Lck-Cre* mice, no *CD4* derepression was observed in peripheral CD8$^+$ T lymphocytes (Figure 4A). This suggested that Runx1 is not required for establishment or maintenance of *CD4* silencing in the cytotoxic T cell lineage. We noticed, however, that although more than 95% of the *Runx1*^{F} alleles were deleted in thymocytes from these mice, deletion was reduced to 23% and 59% of the alleles in mature CD4$^+$ and CD8$^+$ T cells, respectively (Figure 4E). This observation suggests that positive selection favors thymocytes with intact expression of Runx1, and it is hence difficult to determine if this molecule has a role in establishment of *CD4* silencing. Runx1 is unlikely, however, to be required for maintenance of silencing, since a substantial proportion of CD8 lineage T cells have both alleles inactivated. The finding that there is little expression of Runx1 in mature T cells reinforces this conclusion.

Runx1 Regulates CD4 Silencer Function in DN Thymocytes

Although inactivation of the two Runx binding sites in the *CD4* silencer resulted in partial derepression in immature DN thymocytes, the absence of Runx3, which is required for *CD4* silencing in mature CD8 lineage cells, had no effect on CD4 expression in the DN cells (Figure 5A). The *Runx2* mutation also did not affect CD4 expression in DN thymocytes (data not shown). In contrast, in newborn or older *Runx1*$^{F/F}$*/Lck-Cre* mice, there was an increase in the level of CD4 in CD8$^-$ cells presumed to be immature DN thymocytes (Figure 5B and data not shown). We therefore examined CD4 expression on immature thymocytes from 15.5 dpc embryos, as there is little contamination with DP cells at this early stage of T cell ontogeny. In *Runx1*$^{F/F}$*/Lck-Cre* embryos, there was uniform derepression of CD4 in the CD8$^-$TCR$^-$ thymocytes (Figure 5A). In contrast, in thymocytes from *Runx3*-deficient embryos, CD4 was not expressed (Figure 5A). Notably, the level of CD4 expression in the absence of Runx1 was similar to that observed when both Runx sites were absent, which suggests that Runx1 plus other unidentified factors are required to direct full silencing of *CD4* in DN thymocytes.

Variegated Expression of CD8 in the Absence of Runx1

The total number of thymocytes in *Runx1*$^{F/F}$*/Lck-Cre* mice was strikingly reduced compared to control *Runx*$^{+/+}$*/Lck-cre* mice, and there was a marked increase in the proportion of thymocytes with low or absent expression of CD8 (CD4$^+$CD8$^{lo/-}$) compared to control mice (Figure 5B). The CD4$^+$CD8$^{lo/-}$ thymocytes were immature, by virtue of high HSA and low TCRβ surface expression (Figure 5B). Reduction in the total number of thymocytes was first noted in E17.5 thymus of *Runx1*$^{F\text{-}F}$*/Lck-Cre* embryos, a stage at which the CD4$^+$CD8$^+$ subset of T cells normally predominates following rapid expansion of cells with intact pre-TCRs (Figure 5C). The DN thymocyte subset can be further subdivided into four subpopulations, DN1-4, on the basis of surface expression of CD25 and CD44. In *Runx*$^{F/F}$*/Lck-cre* em-

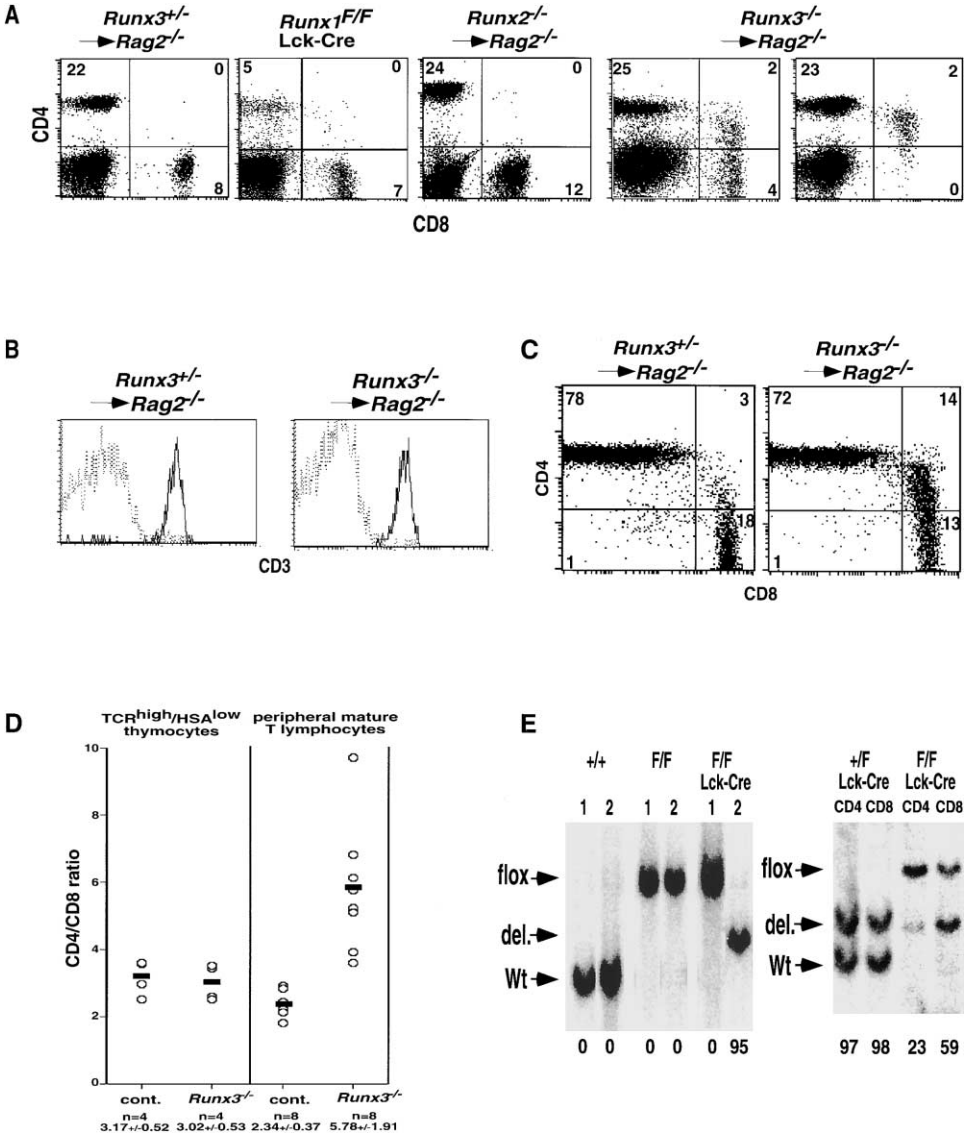

Figure 4. Runx3 Is Required for Establishing Heritable *CD4* Silencing

(A) CD4 and CD8 expression profile on peripheral lymphocytes harboring mutations in each *Runx* gene. Peripheral lymphocytes from *Runx1*$^{F/F}$/Lck-Cre mice or irradiated Rag-deficient host mice reconstituted with fetal liver cells from heterozygous control and *Runx2*$^{-/-}$ or *Runx3*$^{-/-}$ mice were analyzed for their expression of CD4 and CD8 by flow cytometry.

(B) Normal level expression of CD3 on peripheral CD8^{+} lymphocytes (solid line) and CD4^{+}CD8^{+} double-positive (DP) thymocytes (dotted line) from Rag-deficient mice reconstituted with fetal liver cells from *Runx3*$^{-/-}$ mice or heterozygous control littermates.

(C) Derepression of CD4 in mature CD8-lineage thymocytes in the absence of Runx3. Thymocytes from Rag-deficient mice reconstituted with fetal liver cells from *Runx3*$^{-/-}$ or *Runx3*$^{+/-}$mice were stained with anti-TCRβ, anti-HSA, anti-CD4, and anti-CD8 antibodies. The expression profile of CD4 and CD8 in mature (TCR^{high}/HSA^{low}) thymocytes is shown.

(D) Ratio of CD4^{+} to CD8^{+} cells in mature (TCR^{high}/HSA^{low}) thymocytes (left) and peripheral T lymphocytes (right) from mice reconstituted with fetal liver cells from heterozygous control or *Runx3*$^{-/-}$ mice. The peripheral mature CD8^{+} cells include the CD4^{+}CD8^{+} subset from the mutant mice.

(E) Efficiency of Cre-mediated deletion of the *Runx* ^{F} allele in mice expressing the *Lck-Cre* transgene. Southern blot analysis of DNA from tail (1) or thymus (2) is shown in the left image and from purified CD4^{+} or CD8^{+} splenocytes is shown in the right image. Estimated percentage of deletion is shown at the bottom of each lane. The map of the targeted locus is described in Supplemental Figure S2 (available at http://www.cell.com/cgi/content/full/111/5/621/DC1).

bryos, there was an increase in the proportion of DN3 (CD25^{+}CD44^{lo}) thymocytes and a corresponding reduction in DN4 cells (CD25^{-}CD44^{lo}). This result is consistent with impaired β selection in the absence of Runx1 (Figure 5C).

The immature CD4^{+}CD8$^{lo/-}$ subpopulation, which was first observed in E17.5 thymus of *Runx*$^{F/F}$*/Lck-cre* embryos, expressed CD4 at a level equivalent to that in CD4^{+}CD8^{+} DP thymocytes. This level is higher than that observed in DN thymocytes when *Runx1* is mutated or when the entire CD4 silencer is deleted (Figure 5C). Based on their level of CD4 expression and on their kinetics of appearance in the fetal thymus, we conclude that the CD4^{+}CD8$^{lo/-}$ thymocytes constitute a popula-

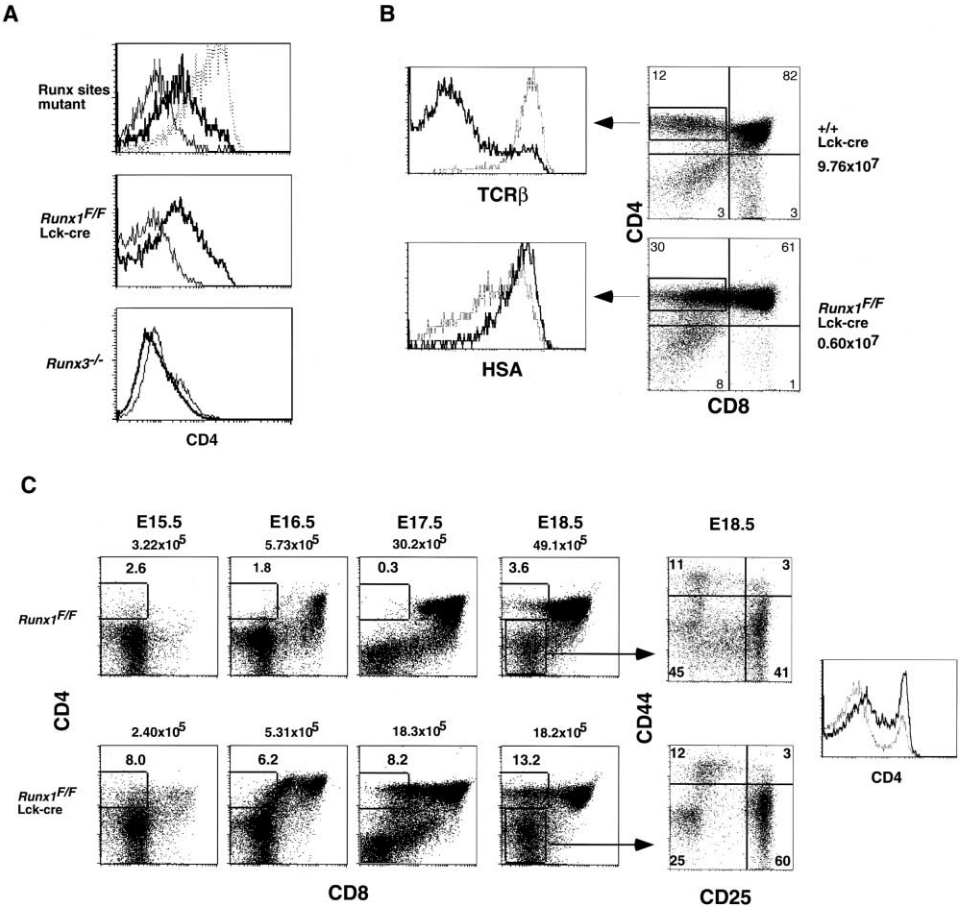

Figure 5. Runx1 Is Required for *CD4* Silencing in DN Thymocytes and for CD8 Expression in DP Thymocytes

(A) CD4 expression in DN subset thymocytes from mice with Runx site mutations in the *CD4* silencer and with mutations of *Runx1* and *Runx3*. CD4 expression is shown in $TCR^-CD3^-CD8^-Thy1^+$ thymocytes. Top image: solid line, bold line, or dotted line represent CD4 expression in wild-type mice, double Runx-site (site 2 and site 2′) mutant mice, and 1–429 *CD4* silencer deleted mice, respectively. In the middle and lower images, thymocytes from 15.5 dpc *Runx1*$^{F/F}$*/Lck-Cre* or *Runx3*$^{-/-}$ embryos were analyzed for CD4 expression. The thin lines and bold lines represent CD4 expression in control and mutant mice, respectively.

(B) Reduction in total thymocyte numbers and appearance of an immature $CD4^+CD8^{lo/-}$ population in *Runx1*$^{F/F}$*/Lck-Cre* mice. The CD4 and CD8 expression profile is from ungated thymocytes from four week-old-mice. Expression of TCRβ and HSA in the gated $CD4^+CD8^{-/lo}$ subpopulation is shown in histograms on the left (light and bold lines represent *Runx1*$^{F/F}$ and *Runx1*$^{F/F}$*/Lck-Cre*, respectively).

(C) Effect of *Runx1*deletion on CD4 and CD8 expression during T cell ontogeny. Embryonic thymi from control (*Runx1*$^{F/F}$) and *Runx1*-deleted animals (*Runx1*$^{F/F}$*/Lck-Cre*) at different stages of development are shown in upper and lower images, respectively. CD44 and CD25 expression profiles of the $CD4^{-/lo}CD8^-$ subpopulation in the thymus from 18.5 dpc embryos are shown at the right and indicate an accumulation of DN3 ($CD25^+CD44^{lo}$) cells in the absence of *Runx1*. CD4 expression on all CD8-negative thymocytes from 18.5 dpc embryos is shown as a histogram in the right image (light and bold lines represent *Runx1*$^{F/F}$ and *Runx1*$^{F/F}$*/Lck-Cre*, respectively).

tion in which CD8 expression is defective. Similar populations were also observed in mice with compound mutations in CD8 enhancers and with impaired function of the BAF chromatin-remodeling complex (Chi et al., 2002; Ellmeier et al., 2002; Garefalaki et al., 2002). The results thus indicate that Runx1 has several key functions in early thymocyte differention. It is required for CD4 silencing in DN thymocytes, for efficient β selection of DN thymocytes, and for upregulation of CD8 as cells differentiate to the DP stage.

Runx3 Is Required for Differentiation of Functional Cytotoxic T Cells

Transcription factors that regulate *CD4* silencing are also good candidates to regulate specialized functions of differentiated cytotoxic T cells. Because Runx3 was found to be required for *CD4* silencing, we wished to determine whether it has a more central role in the specification of cytotoxic T cell function. We therefore compared the ability of $CD8^+$ T cells from wild-type and *Runx3*$^{-/-}$ mice to proliferate in response to allogeneic stimulation and to kill target cells. Splenocytes (H-2^b) recovered from *Rag2*$^{-/-}$ mice reconstituted with wild-type or *Runx3*$^{-/-}$ fetal liver cells were stimulated with irradiated Balb/c splenocytes (H-2^d) for five days, after which $CD8^+$ T cells were purified and mixed with ^{51}Cr-labeled P815 (H-2^d) target cells. In contrast to control cells, *Runx3*$^{-/-}$ $CD8^+$ T cells had a complete loss of specific killing (Figure 6A).

This result could reflect a decrease in the proportion of stimulated allo-reactive $CD8^+$ T cells rather than a defect in effector function. We therefore examined cytotoxic function in a redirected lysis assay, which measures the ability of activated $CD8^+$ T cells to lyse Fc

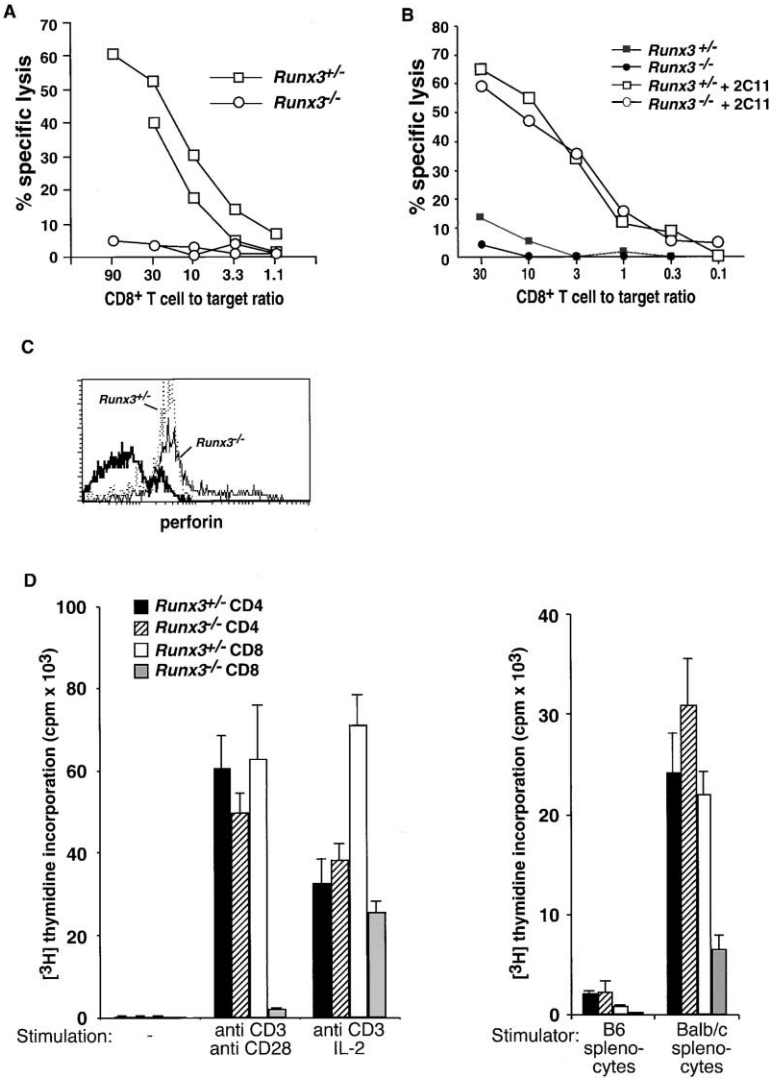

Figure 6. Role of *Runx3* in the Response of Cytotoxic T Cells to Antigen

(A) Reduced allo-specific cytotoxic activity of *Runx3*$^{-/-}$ CD8$^+$ T lymphocytes. Splenocytes derived from Rag-deficient mice reconstituted with control (squares) or *Runx3* $^{-/-}$ (circles) progenitors were stimulated with allogenic cells (H-2^d) in a mixed lymphocytes reaction for five days. Purified CD8$^+$ cells were then mixed with ^{51}Cr-labeled P815 (H-2^d) target cells. Results are shown for two independent experiments.

(B) Normal cytotoxic activity of Runx3-deficient CD8$^+$ T cells in an antibody-mediated redirected cytotoxicity assay. Splenocytes from mice reconstituted with control (squares) or *Runx3* $^{-/-}$ (circles) progenitors were stimulated with immobilized anti-CD3 (5 μg/ml) and anti-CD28 antibody (5 μg/ml) with IL-2 (20 units/ml) for five days. Purified CD8$^+$ cells were mixed with ^{51}Cr-labeled target cells expressing Fc receptor in the absence or presence of anti-CD3 (2C11) antibody.

(C) Normal induction of perforin in CD8$^+$ T lymphocytes from *Runx3*$^{-/-}$ mice. Purified splenic T lymphocytes were stimulated with immobilized anti-CD3 and anti-CD28 antibodies, and perforin induction was analyzed by intracellular staining after 24 hr. Solid line, dotted line, or bold line represent perforin levels in wild-type CD8$^+$ T lymphocytes, *Runx3*-deficient CD8$^+$ T lymphocytes, and wild-type CD4$^+$ T lymphocytes.

(D) Specific reduction of proliferative responses of CD8$^+$ T cells from *Runx3*$^{-/-}$ mice. Purified CD4$^+$ or CD8$^+$ T cells from Rag-deficient reconstituted mice were stimulated with immobilized anti-CD3 antibody in combination with anti-CD28 antibody or IL-2 (left image) for three days or were stimulated with irradiated allogeneic splenocytes (right image) for five days. Proliferative responses were measured by incorporation of ^{3}H-thymidine.

receptor-expressing target cells in the presence of anti-CD3 antibody. In this assay, cells from the mutant mice had normal cytotoxic activity (Figure 6B). Moreover, perforin, which is required for effective cytolytic activity, was induced in Runx3-deficient T cells as well as in control cells after stimulation with anti-CD3 plus anti-CD28 (Figure 6C). However, proliferation in response to anti-CD3 plus anti-CD28 was severely reduced in CD8$^+$ T cells from *Runx3*$^{-/-}$ mice compared to heterozygous littermates (Figure 6D). There was also reduced proliferation in response to stimulation with allogeneic splenocytes (Figure 6D). Remarkably, sorted CD4$^+$CD8$^-$ cells from the mutant mice had normal proliferative responses to all stimuli. These results indicate that *Runx3* is required specifically for CD8$^+$ lineage T cells, but not for T helper cells, to acquire antigen responsiveness.

Discussion

Roles of Runx in *CD4* Silencing

Although it is generally accepted that gene silencing is a prevalent means of gene regulation during development, the mechanisms by which specific genes are repressed or silenced epigenetically are poorly understood and there are few examples of silencers with established in vivo functions (Fisher and Merkenschlager, 2002). We have focused on the well-characterized *CD4* silencer to identify DNA binding proteins required for its function in epigenetic gene regulation during T cell development and have shown that Runx binding sites and cognate Runt domain transcription factors are essential for *CD4* silencing. The original member of this family of transcription factors, *runt*, has been shown to be involved in negative regulation of gene expression during *Drosophila* development (Wheeler et al., 2000). However, whether Runx-mediated repression can also be maintained through epigenetic mechanisms has not been determined, nor have physiologically relevant targets of Runx repressive activity been previously identified in vertebrates.

In this study, we have shown that two Runx binding sites in the *CD4* silencer are essential for full repression of *CD4* transcription in immature DN thymocytes and for establishment of epigenetic silencing in CD4$^-$CD8$^+$ cytotoxic T cells during their development in the thymus. While Runx1 was shown to be required for full repression

in DN cells, Runx3 was found to be required for establishment of epigenetic silencing in CD8-lineage mature thymocytes and T cells, but was not required in DN thymocytes, despite its expression in these cells. These results provide a unique example of a DNA binding transcription factor required for the establishment of epigenetic silencing, and also identify an *in vivo* target for the repressive activity of Runx proteins in vertebrates. In addition, because of the unique properties of the *CD4* silencer, this developmental system provides an opportunity to dissect the distinct functions of Runx proteins in active transcriptional repression and in establishment of epigenetic gene silencing.

In mice with Runx binding site mutations and with loss of function mutations of *Runx1* and *Runx3*, there were distinct patterns of *CD4* derepression at different stages of development. These findings, and previous results with targeted mutations of the silencer (Taniuchi et al., 2002), are consistent with different mechanisms of *CD4* silencing at the two stages of thymocyte differentiation. We propose that Runx proteins provide different repressive functions in immature versus mature thymocytes because of their unique requirement, despite largely overlapping expression, in these cellular subsets.

Mutation of both Runx binding sites resulted in uniform but partial derepression in immature DN thymocytes. In the absence of Runx1, a similar level of derepression was observed in DN cells (Figure 5A). Because both Runx2 and Runx3 are also expressed, the results suggest that Runx1 is the only member of the Runt domain family that can repress at this stage of development, and that Runx1 interacts directly with the two Runx binding sites in the *CD4* silencer. In DN thymocytes, compromised function of the BAF chromatin-remodeling complex resulted in partial CD4 derepression, which was enhanced in mice harboring a mutation in site1 of the *CD4* silencer (Chi et al., 2002). Runx1 may thus be required for recruitment of the BAF complex to the *CD4* locus. Runx1 has also been shown to associate with several corepressor molecules that form complexes with histone deacetylases (HDACs), including Sin3A and Groucho/TLE (Levanon et al., 1998; Lutterbach et al., 2000). Runx1-mediated recruitment of HDACs to the *CD4* silencer may thus result in reversible chromatin modifications, in which the BAF complex is also involved, in DN thymocytes. The partial requirement for Runx sites and Runx1 in *CD4* repression suggests that other proteins, particularly those binding to sites 1 and 3 (Taniuchi et al., 2002), may also recruit HDAC complexes and the BAF complex, resulting in some degree of *CD4* repression in the absence of site 2/2′ function.

Mutation of both Runx binding sites resulted in full derepression of *CD4* in mature CD8-lineage T cells, while variegated *CD4* derepression was observed in *Runx3*$^{-/-}$ thymocytes and T cells. This observation raises the possibility that other Runx proteins can compensate at the time when silencing is established. In addition, the level of derepressed CD4 in Runx3-deficient mice was not as high as in mice with mutations in both Runx binding sites. This suggests that residual binding of factors to sites 2 and 2′, in cells in which epigenetic silencing had failed to be established, can reduce the level of expression. Alternatively, in the absence of Runx3, there could be compromised chromatin modification in the course of establishment of epigenetic silencing in the thymus, and this could result in leaky transcription in mature CD8$^+$ T cells. A similar low level of CD4 derepression was observed in peripheral CD8$^+$ T lymphocytes in which the entire *CD4* silencer was deleted after positive selection, during the establishment phase in the thymus (Zou et al., 2001). This indicated that establishment of epigenetic *CD4* silencing requires a time window for completion of sequential modifications of chromatin structures, as has been shown at the mating type locus of fission yeast (Nakayama et al., 2001).

The repression of *CD4* at the initiation phase of establishment in thymocytes undergoing positive selection toward the cytotoxic lineage is reversible, and its mechanism may hence resemble that in DN thymocytes (Zou et al., 2001). However, it is likely that this process is followed by recruitment of machinery that imposes chromatin modifications required for heritable silencing. A recent study in the *Drosophila* embryo has shown that establishment of repression of *engrailed* (*en*), a well-characterized target gene repressed by *runt*, at the blastoderm stage is independent of DNA binding or association of *runt* with the corepressor Groucho, while maintenance of transcriptional repression during germ band extension requires both of these functions, as well as Groucho and the Rpd3 histone deacetylase (Wheeler et al., 2002). This suggests that maintenance of Runt-directed repression involves alteration of chromatin, although whether the repressed state of *en* is maintained epigenetically is not clear. Alteration of chromatin at the *CD4* locus, which may include histone deacetylation, methylation, and phosphorylation, is likely also initiated by Runx proteins in cells destined for the CD8 lineage, and this is presumably followed by recruitment of epigenetic factors, such as HP-1 and polycomb group proteins.

It remains unclear why Runx1 and Runx3 are differentially required for *CD4* silencing at different stages of development. It is possible that their binding to the silencer is dependent on other factors that, in addition to the common β subunit, associate with specific Runx proteins to affect their DNA binding affinity. For example, at the *TCR*α enhancer, Ets-1 augmented transcriptional activity together with Runx1, but not Runx3, by cooperative binding to target sites (Kim et al., 1999). Thus, the specific function of each Runx at each *cis*-regulatory element might be regulated in part by the specific association with other transcription factors. Within the *CD4* silencer, there are at least two other functional sites, site 1 and site 3, which are distinct from the Runx sites. Double mutations of sites 1 and 3 abolished *CD4* silencing, suggesting that Runx sites are not functional without other functional sites (Taniuchi et al., 2002). It is possible that factors binding to these sites preferentially associate with Runx1 or Runx3, thus regulating their usage. Each of the Runx proteins may also have unique properties required for interactions with repressor or epigenetic machinery.

Roles of Runx in T Cell Differentiation

Since Runx3 is specifically required for lineage-specific *CD4* silencing, this transcription factor could potentially

be a key molecule involved in regulating lineage specificity. Indeed, in its absence, peripheral $CD8^+$ T cells were markedly reduced in number and had a severe proliferative defect. This phenotype is due to the requirement for Runx3 activity on unknown target genes rather than on *CD4*, since $CD8^+$ T cells displayed normal differentiation and function in mice in which the *CD4* silencer was deleted (Zou et al., 2001). The results suggest a requirement for Runx3 in the generation and/or the survival of the cytotoxic T cell population. In light of the function of *Runx3* in epigenetic regulation of *CD4*, it will be interesting to determine whether the defect in cytotoxic T cells is also due to loss of a heritably maintained function in these cells.

We were unable to detect a significant difference in *Runx3* transcript levels between cells of the helper and cytotoxic lineage, indicating that expression of *Runx* genes is not sufficient to induce *CD4* silencing. It is possible that molecules associated with Runx3 or posttranslational modifications of Runx3 regulate lineage specificity of *CD4* gene silencing and CTL function. For example, phosphorylation, which has been reported for Runx1 and Runx2 in response to external stimuli, could be differentially regulated in T cell subsets (Selvamurugan et al., 2000; Tanaka et al., 1996; Wee et al., 2002).

Due to loss of definitive hematopoiesis in the absence of Runx1, it has not been possible to examine its requirement for T cell development. By using Cre-mediated recombination to inactivate *Runx1* in thymocytes, we showed that it is required both for gene activation (*CD8*) and for gene repression (*CD4*) in early T cell development. In addition, impaired β selection observed in *Runx1$^{F/F}$/Lck-Cre* mice is consistent with inefficient rearrangement and activation of *TCR*β genes (I.T., unpublished data).

Inefficient upregulation of CD8 during differentiation of DP thymocytes was observed in three mutant strains: mice deficient for *Runx1*, mice harboring compound mutations in *CD8* enhancers, and mice with impaired function of the BAF chromatin remodeling complex (Chi et al., 2002; Ellmeier et al., 2002; Garefalaki et al., 2002). Various degrees of CD8 expression in cells containing the same mutation in *cis*-regulatory regions suggested stochastic "on" or "off" selection in each cell and involvement of epigenetic mechanisms in maintenance of active transcription. A potential function of Runx1 may be to guide the BAF chromatin-remodeling complex to both the *CD8* and *CD4* loci, to initiate chromatin opening for subsequent recruitment of coactivators or corepressors. The choice of the coregulatory molecules, which is governed by the combination of transcription factors binding to each locus, would determine if genes are repressed or activated.

Epigenetic Gene Regulation by Runx

Our results show that a DNA sequence-specific transcription factor, in this case Runx3, can have an essential role in the establishment of developmental stage-specific and lineage-specific epigenetic gene silencing in vertebrates. Although X inactivation and gene imprinting are well-characterized examples of epigenetic gene silencing, these are established early in embryogenesis and are initiated by RNA (Panning and Jaenisch, 1998; Tilghman, 1999). As Runx proteins have been shown to have pivotal roles in multiple developmental processes across species (Canon and Banerjee, 2000; Karsenty, 2000; Tracey and Speck, 2000), these factors may regulate cell fate determination at developmental branch points, in part through epigenetic activation or silencing of target genes.

Mutations of *RUNX* genes have been shown to be involved in carcinogenesis: *RUNX1* in acute myeloid leukemia (Miyoshi et al., 1991; Osato et al., 1999) and *RUNX3* in gastric cancer (Li et al., 2002). It is possible that Runx factors induce epigenetic silencing of their target oncogenes, so that loss of Runx function results in persistent oncogene activation. In this sense, temporal activation of Runx would be sufficient to permanently inactivate target oncogenes and repress carcinogenesis, as temporal inactivation of Myc resulted in the sustained loss of a neoplastic phenotype (Jain et al., 2002).

Further studies to understand the molecular mechanisms of epigenetic gene regulation by Runx should provide insight into how cells fix their identity during development and in tumorigenesis.

Experimental Procedures

In Vitro Transient Transfection Assay

For construction of CAT reporter plasmids, mutant *CD4* silencer fragments were generated by PCR with appropriate primers, and DNA sequence of these fragments was confirmed. Three copies of each *CD4* silencer fragment were placed in the polylinker site at the 5′ end of pCD4/CD4ECAT vector (Sawada and Littman, 1991). The cDNAs encoding GAL4-DB, Runx1, Runx3, and the Runx1 Runt domain were obtained by PCR with appropriate primers. DNA sequence of these PCR products was confirmed. The cDNAs of GAL4-DB, GAL4-Runx1, GAL4-Runx3, Runx1, Runx3, or the Runx1 Runt domain were placed at the cloning sites of pcDNA3.1 vector (Invitrogen) to generate expression vectors. Transfections of 1200M cells and assays for CAT activity were performed as described previously (Taniuchi et al., 2002).

Antibodies and Flow Cytometry Analyses

All the antibodies used for flow cytometry were purchased from Caltag or Pharmingen unless otherwise mentioned. Anti-mouse perforin antibody was from Kamiya Biomedical. Intracellular staining was performed as described previously (Opferman et al., 1999). Cells stained with fluorescent antibodies were analyzed using FACScan or FACs Calibur flow cytometers and CELL Quest software (BD).

Mice

For targeted mutagenesis of Runx binding sites in the *CD4* silencer, targeting vectors were constructed as described (Taniuchi et al., 2002). Runx2- and Runx3-deficient mice were described previously (Komori et al., 1997; Li et al., 2002). For analysis of T lymphopoiesis in adults, 1–3 × 10^6 fetal liver cells from mutant mice were injected intravenously into sublethally irradiated (5 Gy) Rag2-deficient mice (Jackson Laboratory). Thymocytes or T lymphocytes in the reconstituted host mice were analyzed 8 weeks after transplantation.

To generate mice harboring a *Runx1* allele flanked by *loxP* sites, a targeting vector was constructed with a 5.2 kb XhoI-BamHI 5′ homology region, a 1.3 kb PCR-generated 3′ homology region, and a 4.0 kb BamHI-XhoI fragment in which exon 4 was flanked by *loxP* sequences. 30 μg of NotI-linearized vector was electroporated into E14 embryonic stem cells and G418/ganciclovir double-resistant colonies were screened for homologous recombination by PCR. Positive clones were subjected to Southern analysis to confirm integration of the third *loxP* site. The positive clones were transiently transfected with the Cre recombinase expression vector pMC-Cre (Gu et al., 1993) to delete the neomycin resistance cassette (*neo^r*). After deletion of *neo^r*, ES cells were injected into C57BL6 blastocysts to generate chimeric mice.

Yeast One-Hybrid Screening

Three copies of the wild-type *CD4* silencer fragment (sequence 131–265) were cloned into multiple cloning sites of the pHISi-1 or pLacZi vectors (Clontech) to make reporter gene constructs. The yeast strain, YM4271 from Clontech, was transformed with these plasmids to generate a dual reporter strain, 131–265/wt.

A cDNA library was prepared from mouse thymus mRNA with the COPY kit (Invitrogen) and cloned into pACT2 vector (Clontech). Screening of the cDNA library, the β-galactosidase assay and isolation of cDNA clones from yeast were performed according to the manufacturer's instructions. Briefly, after introduction of the cDNA library into the yeast strain 131–265/wt, cells were plated in the absence of leucine/histidine plus 35 mM 3-aminotriazole (3-AT). Positive transformants were analyzed for β-galactosidase activity. The cDNA clones recovered from transformants expressing high β-galactosidase activity were reintroduced into the 131–265/wt strain to confirm reproducible activation of the reporter gene. Out of 5×10^6 cDNA clones screened, six independent clones activated the reporter genes through the 131–265 *CD4* silencer fragment. These six cDNA clones were introduced into a second set of yeast reporter strains containing 212/wt, 212/Mut3-2, 212/Mut3-3, or 212/Mut3-5 *CD4* silencer fragments in the pLacZi vector. Only one clone, T-1, showed site 2-dependent reporter gene activation.

T Lymphocyte Stimulation and Culture

For the proliferation assays and a mixed lymphocyte reaction, splenocytes were harvested from the Rag-deficient host mice reconstituted with *Runx3*$^{-/-}$ fetal liver cells and incubated with anti-CD8 magnetic microbeads (Miltenyi Biotec). For $CD8^+$ cell purification, labeled cells were purified with 2 rounds of positive selection according to the manufacturer's instructions. For $CD4^+CD8^-$ cell purification, $CD8^+$ cells were depleted with negative selection and then $CD8^-$ cells were labeled with anti-CD4 magnetic beads and purified with positive selection. The purity of the resulting populations was assayed by flow cytometry and was about 95%. A total of 5×10^4 of $CD4^+CD8^-$ cells and $CD8^+$ T cells were stimulated with 2 μg/ml of immobilized anti-CD3 antibody (Pharmingen) in combination with 2 μg/ml anti-CD28 (Pharmingen) or 20 units/ml of recombinant murine interleukin-2 (Roche) for 3 days or stimulated with 1×10^5 irradiated (20 Gy) Balb/c splenocytes for 5 days. Cultures were pulsed with [^{3}H]thymidine during the last 4 hr.

Cytotoxicity Assays

Splenocytes (H-2^b) from reconstituted host mice were stimulated with irradiated allogenic Balb/c (H-2^d) splenocytes in the presence of mIL-2 (20 units/ml). Five days later, $CD8^+$ cells were purified with MACS using CD8-beads (Miltenyi Biotec), and were mixed with ^{51}Cr-labeled target cells, P815 (H-2^d). For the redirected cytotoxicity assay, splenocytes from host mice were stimulated with 5 μg/ml of immobilized anti-mCD3 antibody and 5 μg/ml of anti-mCD28 antibody with mIL-2 (20 units/ml) for five days. Purified $CD8^+$ cells were mixed with ^{51}Cr-labeled P815 cells expressing Fc receptor with anti-CD3 antibody.

Real-Time RT-PCR Analysis

Each T cell subset was purified by sorting on a Coulter Elite cell sorter. First strand cDNA was synthesized from 1 μg of total RNA with SuperScript (Invitrogen). PCR was performed with an iCycler (Bio-Rad) and SYBR Green PCR reagent (Applied Biosystems). Primers for *Runx 1*, *Runx2*, *Runx3*, *cbf*β, *CD4*, and β *actin* are available in the Supplemental Data (available at http://www.cell.com/cgi/content/full/111/5/621/DC1).

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